Planta (Berl.) 118, 183—193 (1974)

Ultrastructural Studies of the Aggregation and Fusion of Plant Protoplasts

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Received April 2, 1974

Summary. Electron microscopy has been used to assess the efficiency of four different techniques as potential methods for fusing isolated plant protoplasts. Protoplast aggregation has been achieved by the use of an antiserum, high pH, polyethylene glycol 6000 or poly-L-lysine. The ultrastructural characteristics of each treatment are described. Fusion of adjacent surface membranes does not necessarily follow aggregation, and has not been observed in protoplasts aggregated by antisera. The other three methods all result in some degree of fusion, depending upon the state of the protoplasts and the temperature of the experiment. Fusion is favoured by a high density of cytoplasm in the protoplasts and by higher temperatures. The methods are discussed in terms of their likely applicability as a practical fusion technique.

Introduction

One of the general goals for research with isolated plant protoplasts is their interspecific fusion. Several methods for aggregating protoplasts have been proposed (Power *et al.*, 1970; Potrykus, 1971; Hartmann *et al.*, 1973; Keller and Melchers, 1973; Kao and Michayluk, 1974). Aggregation is a necessary prequisite for fusion; other desirable properties of potential fusion techniques include minimal loss of viability of the protoplasts, general applicability and as high a rate of fusion as is compatible with these features.

The first method to be published for the fusion of enzymatically isolated protoplasts involved the use of sodium nitrate at high concentration to discharge the surface membrane (Power *et al.*, 1970). This method has been used successfully in conjunction with selective media for the production of a hybrid of tobacco (Carlson *et al.*, 1972). It has been studied ultrastructurally by Davey and Short (1973). In general this method suffers from low efficiency and a poor effect on protoplast viability, as well as low general applicability (Potrykus, 1973).

More recent methods of aggregation have shown greater promise in regard to side-effects on the protoplasts. However, no ultrastructural study has yet confirmed their efficiency as fusion techniques and light

microscopy gives inadequate information concerning early fusion events (Davey and Short, 1973). The work to be reported here is a study of the aggregation of tobacco mesophyll protoplasts by four different agents; a specific antiserum, high pH, polyethylene glycol and, by analogy with lysozyme (Potrykus, 1973), the polycation poly-L-lysine (PLL). The methods are assessed in terms of their side effects and efficiency as fusion techniques.

Materials and Methods

a) Protoplast Isolation. This was carried out as described by Motoyoshi *et al.* (1973). Protoplasts maintained overnight after an aggregation treatment were suspended in the simplified medium containing antibiotics used in virus studies (referred to below as "incubation medium"). All protoplasts were washed free of enzymes by a cycle of three low speed centrifugations in 0.7 M mannitol before use. This is claimed (Kao and Michayluk, 1974) to reduce interspecific fusion in a mixed protoplast population. We considered it important to obtain a uniform starting preparation of protoplasts for different treatments, and found no evidence for reduction of fusion rate between tobacco protoplasts.

b) Preparation and Use of Antiserum. Whole protoplasts, freshly isolated, were suspended at 1×10^6/ml in the saline solution of Hartmann *et al.* (1973). Rabbits were given 3 weekly 1 ml injections of such a suspension in the marginal ear vein. Animals were bled from the ear 1 week after the final injection and the antiserum was prepared as described by Campbell *et al.* (1970). For some experiments the gamma globulin was prepared by three precipitations from 33% saturated ammonium sulphate (Campbell *et al.*, 1970). In use heated whole serum (Hartmann *et al.*, 1973) or purified gamma globulin was diluted stepwise with 0.7 M mannitol. Under our conditions aggregation occurred up to 1/16 or 1/32 concentration. For electron microscope studies a dilution of 1/8 was used. Protoplasts were suspended in diluted serum and samples fixed at intervals. (See Results section.)

c) Aggregation Using High pH. This was carried out exactly was described by Keller and Melchers (1973). For details of sampling for electron microscopy, see Results section.

d) Aggregation Using Polyethylene Glycol 6000. Protoplasts were suspended at $3–5 \times 10^5$/ml in 0.7 M mannitol, and an equal volume of a solution containing 450 g/l polyethylene glycol 6000 (PEG), 0.1 M glucose and 5 mM calcium chloride was added with gentle stirring. After 15 or 30 min, the resulting suspension was diluted with an equal volume of incubation medium. After another 30 min, a further equal volume of incubation medium was added and the protoplasts then washed in incubation medium by centrifugation. Samples for electron microscopy were taken at all stages. The high viscosity of the PEG solution necessitated the addition of a large excess of fixative to the undiluted samples. Care was taken not to centrifuge unfixed protoplasts in the presence of undiluted PEG solutions since this led to formation of large clumps of protoplasts. Experiments were run at 25 °C and 37 °C.

e) Aggregation Using Poly-L-Lysine. Protoplasts were suspended at $1–3 \times 10^5$/ml in citrate-buffered mannitol at pH 5.3 containing poly-L-lysine (PLL) at 10, 25, or 50 μg/ml. Samples for electron microscopy were taken after 10 or 30 min incubation at 25 °C, 30 °C or 37 °C.

f) Electron Microscopy. This was carried out exactly as previously described (Burgess *et al.*, 1973a), but using mannitol as osmoticum in the fixative.

Results

a) Aggregation with Antisera. Aggregation began within 15 min of suspending the protoplasts in diluted serum, and specimens were fixed for electron microscopy at 30 min, 1 h and 16 h. The appearance of protoplasts treated for up to one hour with serum was quite normal, however after 16 h in heated whole serum many protoplasts were badly fixed and apparently moribund. Overnight incubation with equivalent dilutions of the purified gamma globulin did not have this effect.

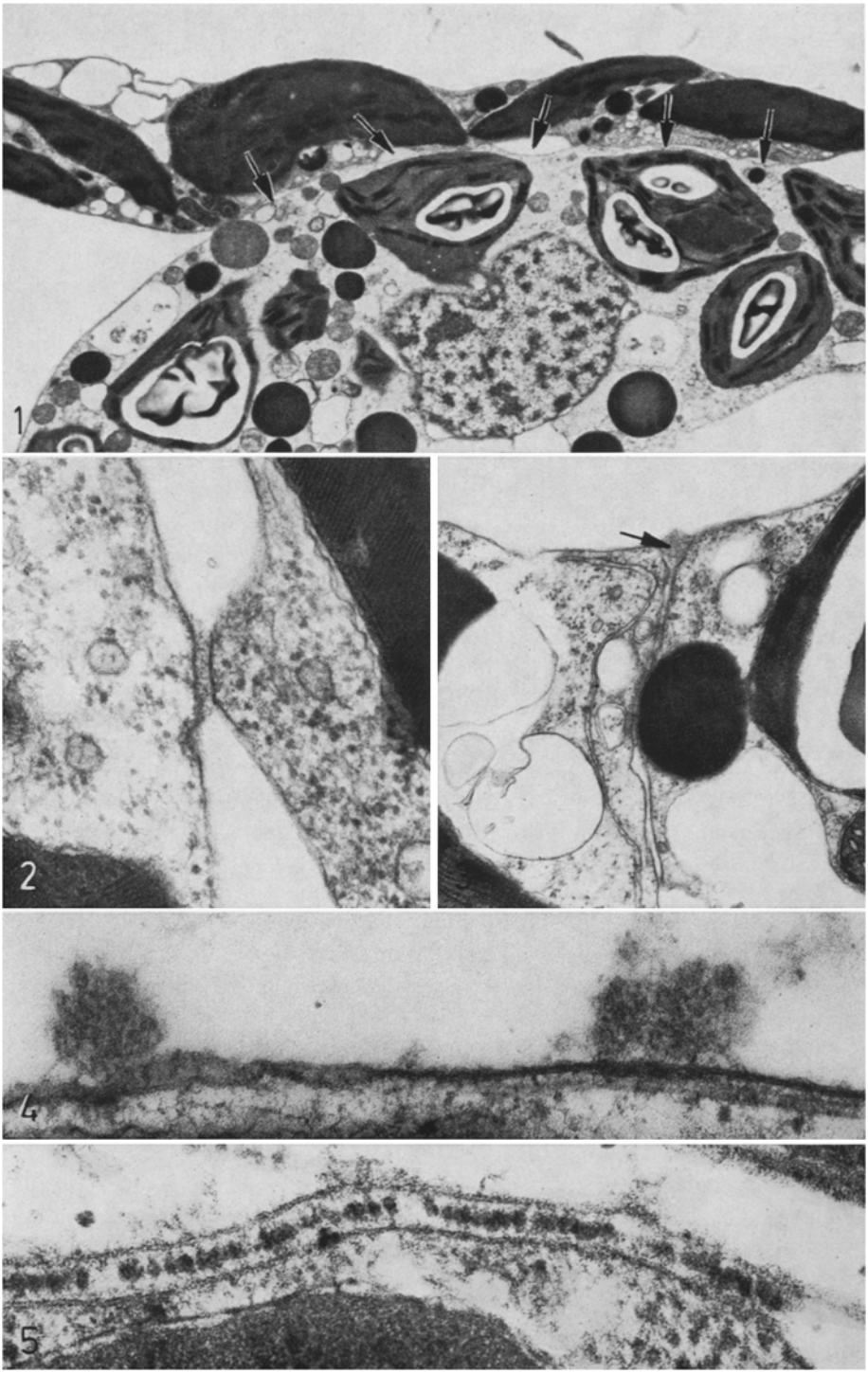
Adjacent protoplasts were associated for long distances (Fig. 1). In some regions the plasma membranes were closely parallel whilst in other regions close approach was achieved only locally (Fig. 2). At such points of approach an amorphous stained material appeared to separate the two surface membranes (Fig. 2). Clumps of similar material also appeared frequently at the point of divergence of adjacent membranes (Fig. 3), and also on those parts of the protoplast surface which were not involved in aggregation (Fig. 4).

After overnight incubation in the purified gamma globulin the surface membranes of both aggregated and non-aggregated protoplasts were frequently studded with particles arranged in rows (Fig. 5). Protoplasts exposed to diluted whole serum for one hour, then kept overnight in incubation medium appeared moribund and were not aggregated.

No convincing evidence for fusion between aggregated protoplasts was obtained under any of the conditions studied.

b) High pH in the Presence of Calcium Ions at 37 °C. Aggregation at pH 10.5 began within 5–10 min, and resulted in the formation of very large clumps containing many protoplasts as well as smaller clumps of two to five protoplasts. Specimens were fixed after 10, 15, 30 and 45 min in the buffered mannitol, and also after an overnight period in incubation medium following a 15 min treatment at high pH.

The general appearance of the protoplasts depended upon the size of the aggregate of which they were a part. Larger clumps were very badly preserved, and this appeared to be due to extensive damage to the surface membranes. Single protoplasts and small groups of two or three were more normal in structure. Aggregation appeared to take place by a series of very close points of contact along the surface which in many cases became sites of fusion (Fig. 6). This type of localised formation of fusion channels resulted in the formation of enclosed vacuoles within the joined cytoplasm of the fusion body. Such vacuoles initially marked the course of the surface of the original participants in the fusion (Fig. 7). Frequently such vacuoles contained closed membraneous units filled with ribosome-like particles (Fig. 8). The dispersal of these vacuoles it not impossible to illustrate with confidence since advanced stages of induced fusion either



Figs. 1—5

can not be recognised or not distinguished from "spontaneous" fusion occurring at a much lower frequency during protoplast preparation. Recognisable fusion channels between protoplasts were a common feature of specimens fixed even after only ten minutes in the high pH medium.

Protoplasts kept overnight in incubation medium following a 15 min exposure to high pH retained a normal morphology and judged by structural criteria, were viable in many cases (Burgess *et al.*, 1973a).

c) Aggregation in Media Containing PEG. Aggregation proceeded more slowly than either of the previous methods, possibly due to the high viscosity of the medium in which the protoplasts were suspended. Under the conditions described above most of the aggregates formed comprised two or three protoplasts and large clumps were avoided. In general the effects of the treatment on the internal structure of the protoplasts appeared very slight. Specimens were fixed whilst in PEG medium, or after dilution once, or after washing.

The ultrastructural pattern of aggregation was different from either of the previously described treatments. Adjacent membranes were often closely associated with no intervening space or structure over long distances (Fig. 9). In other sections the spacing between the adjacent surfaces was irregular, with local points of contact. Points of contact often involved a marked distortion of one or both plasma membranes, and gave the impression that the membranes were both fluid and "sticky" (Fig. 10). Such behaviour was found in all specimens. Fusion was only observed with certainty in protoplasts which had been diluted or diluted and washed after the PEG treatment. Very few sections showed fusion bodies, and it appeared that at 25 °C only protoplasts with rather densely stained cytoplasm underwent fusion (Fig. 11), whereas protoplasts in which the cytoplasm was less dense or vacuolate (Fig. 9) did not.

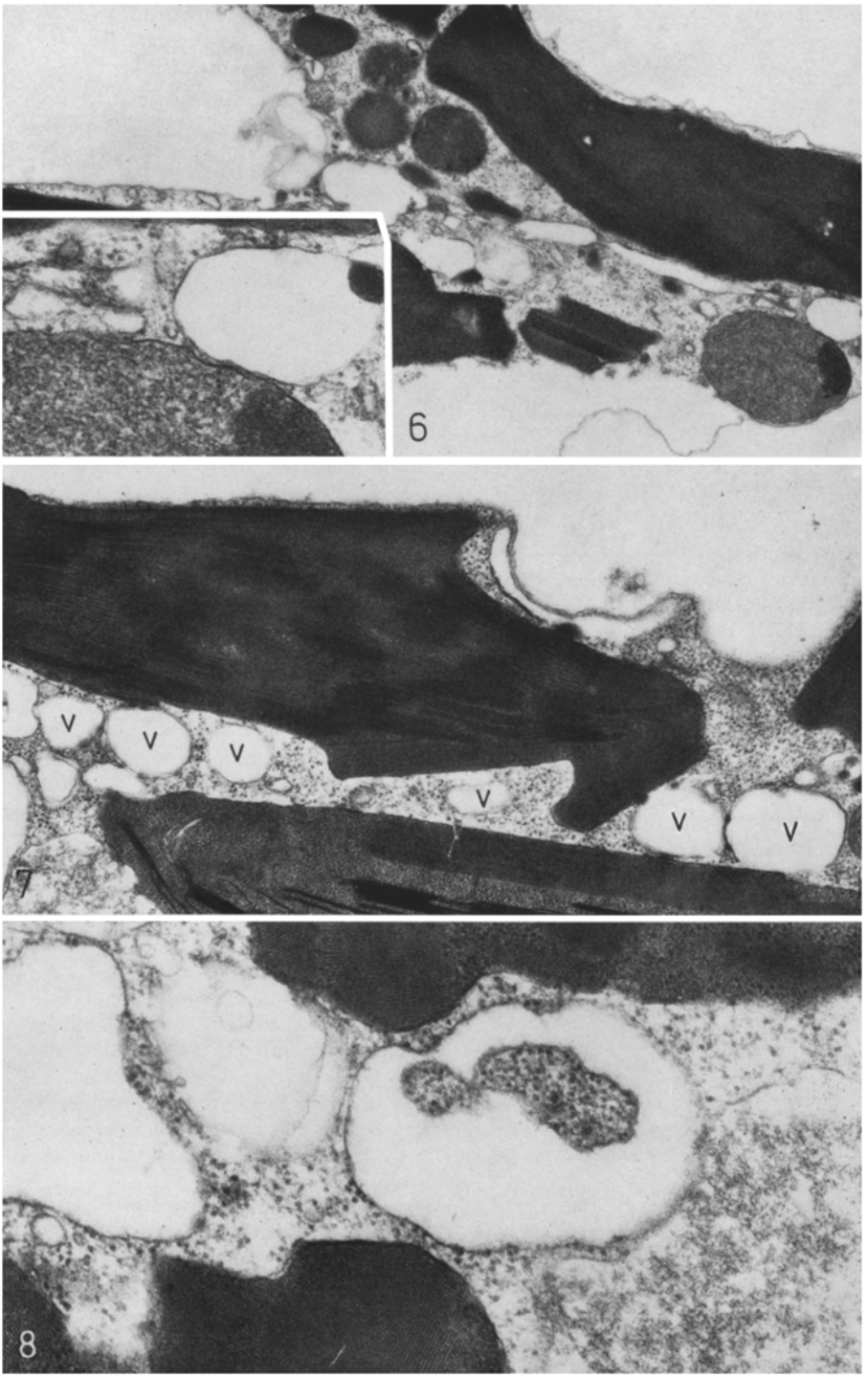
Fig. 1. Section through the junction between two aggregated protoplasts after one hour in heated whole serum diluted to 1/8. Regions of apparently close association of the surfaces are separated by non-associated regions (arrows). There is no effect on internal morphology. × 7000

Fig. 2. A detail from a similar specimen to that shown in Fig. 1. At points of closest approach the surface membranes are separated by a layer of amorphous material which is about three times the thickness of each plasma membrane. × 60000

Fig. 3. Section through the edge of a junction between two aggregated protoplasts fixed as for Fig. 1. A plug of amorphous stained material is visible at the outermost edge of the junction (arrow). × 24000

Fig. 4. Part of the surface of a protoplast exposed to diluted heated whole serum for 30 min, and which is not associated with other protoplasts. Clumps of amorphous stained material are visible attached to the plasma membrane. × 100000

Fig. 5. Details of a pair of adjacent surface membranes of protoplasts incubated overnight with a diluted purified gamma globulin fraction of antiserum. Regions of the membrane as shown are studded with stained particles. × 120000



Figs. 6—8

In a further series of experiments the protoplasts were kept at 37 °C in all solutions prior to fixation. This resulted in a very striking increase in the rate of fusion between adjacent protoplasts, and apparently overcame the barriers to fusion between the less dense or damaged protoplasts. Protoplasts appeared to survive treatment well at the higher temperature.

d) Aggregation by PLL. This polycation is less toxic than poly-L-ornithine (Watts, unpublished observations), and caused aggregation when used at 25 or 50 μg/ml. Regions of close association over considerable distance were observed, as well as point contact (Fig. 12). Protoplasts suffered some surface damage and often contained many small vacuoles as a result of this (cf. Burgess *et al.*, 1973 b). Fusion was not seen frequently at any time at 25 °C; however in samples which were exposed to PLL for 30 min at 30 °C some evidence was obtained for the fusion of closely adpressed membranes (Fig. 13). Exposure of protoplasts to 25 or 50 μg/ml of PLL at 37 °C resulted in extensive damage and death of the protoplasts.

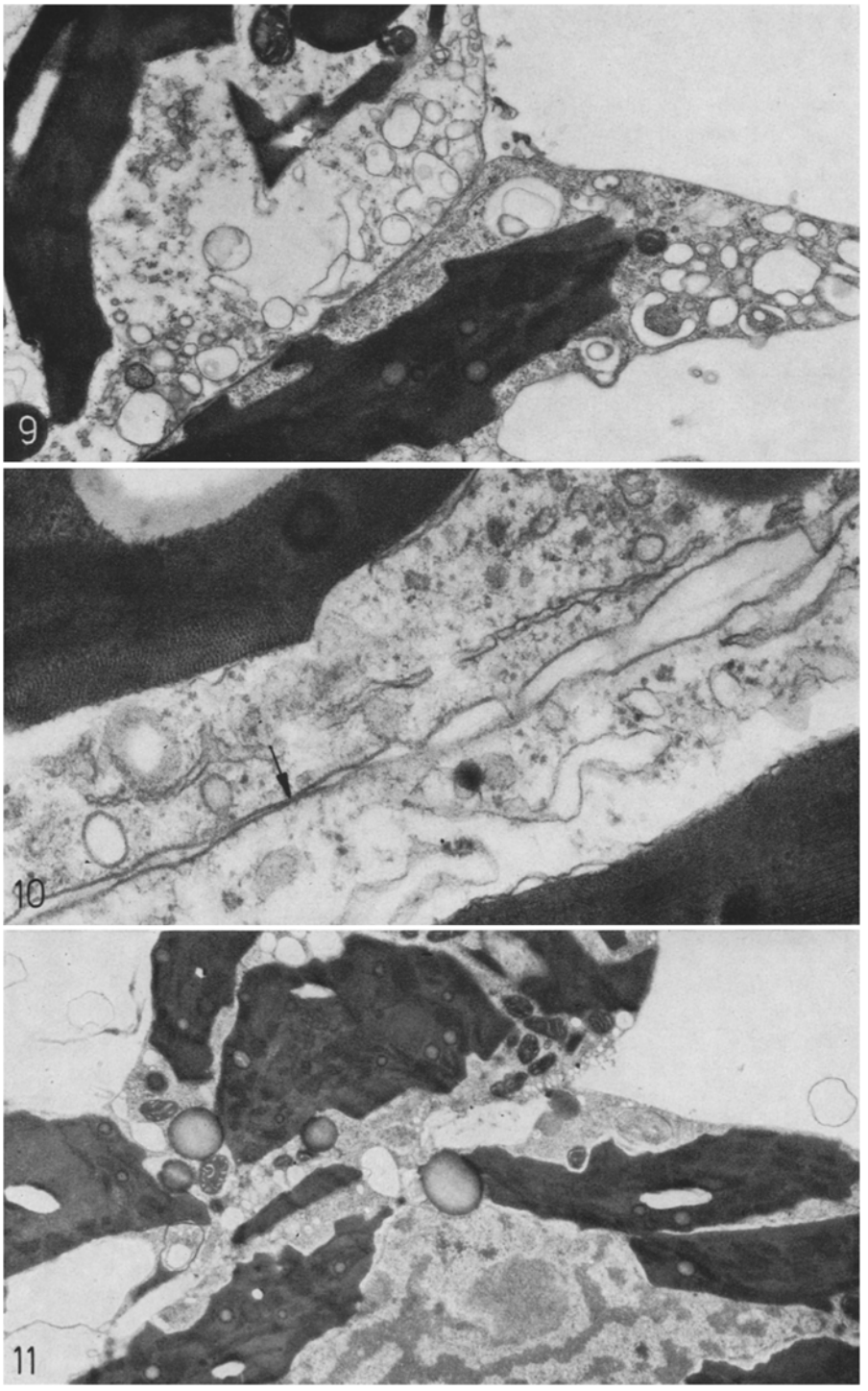
Discussion

Several general conclusions may be drawn concerning techniques for aggregation and fusion of protoplasts from this study of the early events of the process. It is clear that visible clumping of protoplasts may be achieved by agents which are unlikely to increase the rate of fusion between adjacent units. This is exemplified by the use of antiserum, where the agglutination mechanism (Hartmann *et al.*, 1973) can be seen to prevent the approach of the surface membranes at a molecular level. However, close approach of two plasma membranes may not on its own be a sufficient precondition for their fusion. Both PEG and PLL cause an apparently very tight conjunction of surface membranes over extensive areas, but fusion bodies are only seen when other factors are favourable as discussed below. Conversely, treatment at high pH at 37 °C gives a very high rate of fusion, within aggregates which are held in contact at quite localised points.

Fig. 6. Section through a pair of protoplasts incubated for 15 min with a pH 10.5 glycine buffer with 50 mM $CaCL_2$. The protoplasts show local touching and formation of small fusion channels (inset). Membrane damage is evident at the outermost edge of the junction. × 15000. Inset × 48000

Fig. 7. Section through the cytoplasm of a fused protoplast exposed to high pH for 30 min. The small vacuoles (*v*) are residual parts of the plasmalemma, separated by fusion channels. × 25000

Fig. 8. Section through a plasmalemma vacuole in the cytoplasm of a recently fused protoplast aggregate. The vacuole contains a membrane-bound body filled with ribosomelike particles. × 50000



Figs. 9—11

Two other factors are of apparent importance in determining whether a fusion body is formed from aggregated protoplasts. The first is the condition of the protoplasts themselves. In the case of comparatively mild and inefficient fusion techniques such as PEG or PLL treatment at room temperature, fusion only occurs between densely cytoplasmic protoplasts. Those which are less dense or contain small vacuoles in large numbers, possibly due to damage during preparation or treatment (Burgess *et al.*, 1973 b) are not observed to fuse under such conditions. Paradoxically exposure to high pH at 37° C itself results in considerable damage to protoplasts, especially those in large clumps. It is our impression that a practical technique must balance the degree of damage with the degree to which the surface membranes are modified to predispose them to fusion; the high pH method is clearly more drastic in its effects than either PEG or PLL. The reason for vacuolate protoplasts failing to fuse under marginal conditions such as PEG or PLL treatment at low temperature is not known, but it is consistent with earlier observations that meristematic-type protoplasts fuse far more readily than those from tissue containing vacuolate cells (Power *et al.*, 1970). An extreme example of this effect is provided by the very high fusion rates achieved between meiotic protoplasts without any artificial treatment other than the use of cell-wall degrading enzymes (Ito, 1973).

The effect of temperature upon fusion rates using the high pH method has been discussed by Keller and Melchers (1973), and is known from work with animal cells (Ahkong *et al.*, 1973). Our results confirm these findings. Protoplasts treated with PEG or PLL at room temperature (25 °C) aggregate tightly but do not fuse readily unless the members of the aggregate are comparatively undamaged as already discussed. Raising the temperature to 30 °C or to 37 °C in the case of PEG treatment increases the frequency of fusion dramatically. Protoplasts exposed to PLL are unstable at 37 °C. No attempt has been made to quantify this effect of temperature in the present study, but is clearly an important consideration for any practical technique. The behaviour of protoplasts in PLL again demonstrates the need for compromise between efficiency of fusion and associated side effects which reduce protoplast viability.

Fig. 9. Section through part of a protoplast aggregate exposed to PEG solution at 25 °C for 15 min, then diluted once and fixed after a further 15 min. The surface membranes are closely and uniformly associated, but no fusion has occurred. One protoplast is considerably more densely cytoplasmic than the other. × 15000

Fig. 10. Part of a pair of adjacent surface membranes in a protoplast aggregate formed during 15 min incubation in PEG. The membranes can approach one another very closely (arrow) and also show a high degree of distortion in local regions of apparently strong adhesion. × 60000

Fig. 11. Section through the junction between two very densely cytoplasmic protoplasts fixed as in Fig. 9. Extensive fusion has occurred, giving rise to many very small vacuoles in the joint cytoplasm. × 7500

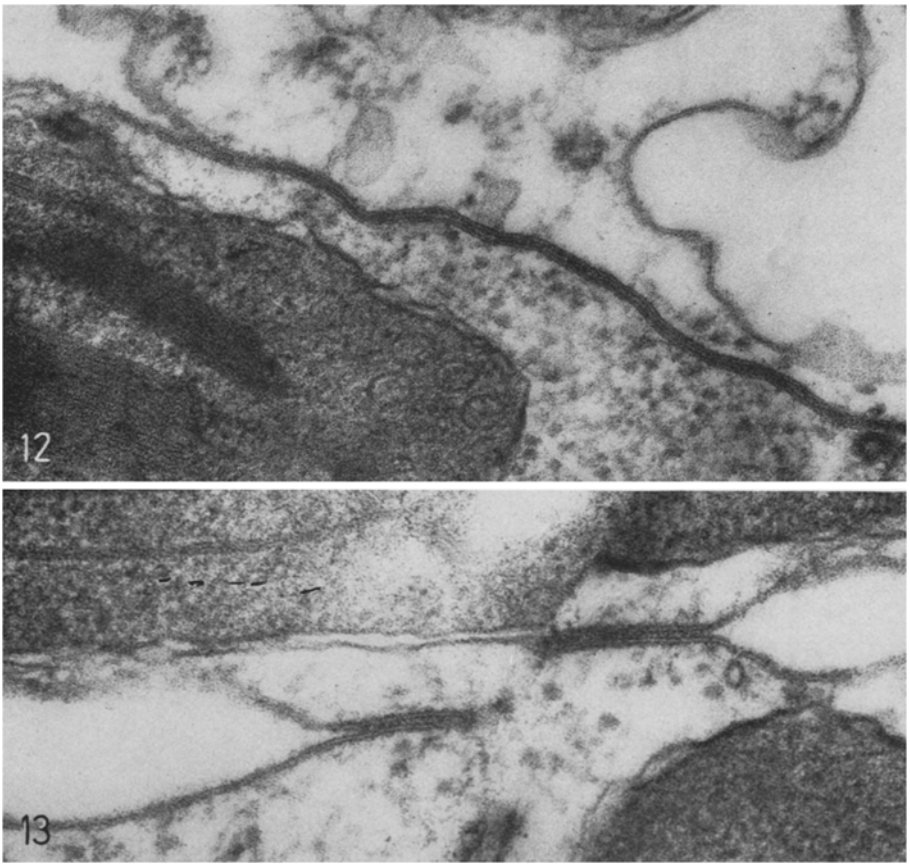


Fig. 12. Section through part of a pair of adjacent surface membranes of protoplasts aggregated in 25 μg/ml PLL for 30 min at 30 °C. The membranes are very closely associated with a separation of about the width of one plasma membrane, or less. × 100000

Fig. 13. Similar to Fig. 12. Under these conditions it appears that small fusion channels may form within regions of tight association of adjacent membranes. × 120000

Clearly the test of any fusion method is the regeneration of a product of fusion. Our comments concerning "viability" are limited to a subjective appraisal of the fine structure of the protoplasts following treatment and may not represent an accurate assessment of long-term survival possibilities. With this reservation we conclude that PEG treatment offers the best hope of a practically useful technique of those we

have examined. The rather simpler conditions we have used than those originally published (Kao and Michayluk, 1974) do not appear adversely to affect the results. Treatment at a temperature of 30° or 37 °C produced a marked improvement in the efficiency of the method.

References

Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I., Tampion, W., Verrinder, M., Lucy, J. A.: Chemically-induced and thermally-induced cell fusion: lipid-lipid interactions. Nature (Lond.) New Biol. **242**, 215–217 (1973)

Burgess, J., Motoyoshi, F., Fleming, E. N.: Effect of poly-L-ornithine on isolated tobacco mesophyll protoplasts: Evidence against stimulated pinocytosis. Planta (Berl.) **111**, 199–208 (1973b)

Burgess, J., Watts, J.W., Fleming, E. N., King, J. M.: Plasmalemma fine structure in isolated tobacco mesophyll protoplasts. Planta (Berl.) **110**, 291–301 (1973a)

Campbell, D. H., Garvey, J. S., Cremer, N. E., Sussdorf, D. H.: Methods in immunology. New York: Benjamin 1970

Carlson, P. S., Smith, H. H., Dearing, R. D.: Parasexual interspecific plant hybridisation. Proc. nat. Acad. Sci. (Wash.) **69**, 2292–2294 (1972)

Davey, M. R., Short, K. C.: The isolation, culture and fusion of legume and cornflower leaf protoplasts. In: Protoplastes et fusion de cellules somatiques végétales, p. 437–453. Paris: CNRS 1973

Hartmann, J. X., Kao, K. N., Gamborg, O. L., Miller, R. A.: Immunological methods for the agglutination of protoplasts from cell suspension cultures of different genera. Planta (Berl.) **112**, 45–56 (1973)

Ito, M.: Studies on the behaviour of meiotic protoplasts II. Induction of a high fusion frequency in protoplasts from liliaceous plants. Plant & Cell Physiol. **14**, 865–872 (1973)

Kao, K. N., Michayluk, M. R.: A method for high-frequency intergeneric fusion of plant protoplasts. Plant (Berl.) **115**, 355–367 (1974)

Keller, W. A., Melchers, G.: The effect of high pH and calcium on tobacco leaf protoplast fusion. Z. Naturforsch. **28c**, 737–741 (1973)

Motoyoshi, F., Bancroft, J. B., Watts, J.W., Burgess, J.: The infection of tobacco protoplasts with cowpea chlorotic mottle virus and its RNA. J. gen. Virol. **20**, 177–193 (1973)

Potrykus, I.: Intra and interspecific fusion of protoplasts from petals of *Torenia baillonii* and *Torenia fournieri*. Nature (Lond.) New Biol. **231**, 51–58 (1971)

Potrykus, I.: Isolation, fusion and culture of protoplasts of Petunia. In: Yeast, mould and plant protoplasts, p. 319–332. London: Acad. Press 1973

Power, J. B., Cummins, S. E., Cocking, E. C.: Fusion of isolated plant protoplasts. Nature (Lond.) **225**, 1016–1018 (1970)